Anti-Platelet and Membrane-Rigidifying Flavonoids in Brownish Scale of Onion

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The bio-activity of the brownish scale of onion (*Allium cepa*) was studied together with identifying the active components and addressing the mode of action. A crude MeOH extract (0.5–1.0 mg/ml) showed the inhibitory effects on human platelet aggregation induced by collagen, adenosine 5′-diphosphate (ADP), thrombin and epinephrine. The anti-platelet extract (1.0 μg/ml) rigidified liposomal membranes by acting on the hydrocarbon core more intensively than the surface of membrane lipid bilayers. Serial solvent extractions and chromatographic purifications provided four isolates which were structurally identified as different quercetin dimers (1 and 2), quercetin (3) and quercetin-4′-glucoside (4). The flavonoidal components 1, 3, 2 and 4 (0.5–2 mM) inhibited collagen-induced platelet aggregation in increasing order of intensity. More active 1 and 3 (2 mM) also dissociated the aggregates produced by ADP. The anti-platelet flavonoids (0.25–10 μM) acted on liposomes of the lipid composition resembling human platelets to cause membrane rigidification which was greatest in the order of 1, 2, 3 and 4. The interaction with membrane lipids to modify membrane fluidity appears to be partly responsible for the anti-aggregatory and disaggregatory effects on human platelets. Although the inedible scale of onion is usually regarded as waste, it has the possibility to be a medicinal resource.

Key words —— onion, brownish scale, flavonoid, anti-platelet activity, membrane rigidification

INTRODUCTION

In recent years, there is increasing attention to the health benefits of foods, beverages, spices and seasonings beyond their nutritional, tasting and flavoring significance.[1)] Especially, plant-originating foodstuffs like green tea, soy bean, grape and their products have been recognized to exhibit the preventive and/or therapeutic effects on various diseases.[2–5)] If the concept of "utilization of the functionality of foodstuffs" is expanded to the inedible parts of plant foods dumped in everyday life, the medicinal utility may be produced from such waste.

Among functional plant foods, onion (*Allium cepa*) shows a variety of pharmacological effects such as growth-inhibition of tumor and microbial cells, reduction of cancer risk, scavenging of free radicals and protection against cardiovascular diseases, which are attributed to bio-active diallyl sulfide and flavonol components.[3,6)] Although onion bulbs are known to have an exceptionally high content of flavonols, the inedible scale has scarcely been focused with regard to the potent bio-activity compared with the edible inner bulb. In the course of screenings for onion components, the brownish scale as waste was found to contain flavonoids other than quercetin. We report here their structures, inhibitory effects on human platelet aggregation and one of possible anti-platelet mechanisms.

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MATERIALS AND METHODS

Preparation of Onion —— The brownish scales of onion (600 g) obtained from commercial outlets were air-dried, powdered and extracted with MeOH (5 l) at room temperature for 6 days. The MeOH extract (56 g) was subjected to silica gel chromatographic fractionation eluted with $CHCl_3$-MeOH increasing polarity. The $CHCl_3$-MeOH (20 : 1, v/v) fraction was chromatographed on Sephadex LH 20 and eluted

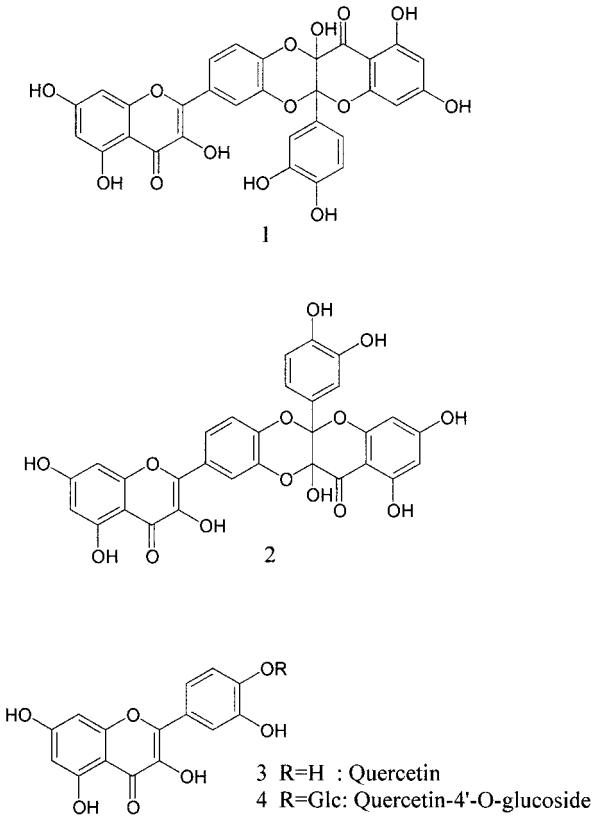

Fig. 1. Structures of Flavonoids Isolated from Brownish Scale of Onion

with MeOH to yield 1 (60 mg), 2 (56 mg) and impure quercetin (3, 3.9 g) which was finally purified by Sephadex LH 20 chromatography eluted with a mixture of EtOH and *n*-hexane (9 : 1, v/v). The $CHCl_3$-MeOH (10 : 1, v/v) fraction was similarly chromatographed to yield quercetin-4′-glucoside (4, 2.8 g). On the basis of spectral analysis (UV, ^1H and ^{13}C NMR, negative ion FABMS spectra, *etc.*), 1 and 2 were identified as different quercetin dimers (Fig. 1). Their chemical structures agreed with those reported by Hirose *et al.*[7] They were isolated in a mixture of stereoisomers that gave a single spot on TLC and a single peak on HPLC analysis.

Synthesis of 1 and 2 —— Since 1 and 2 were presumed to be formed from quercetin through radical scavenging reaction, we developed their facile synthesis in which 3 was allowed to react with free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). A solution of DPPH (2.6 g, 6.6 mmol) in MeOH was mixed with 3 (1.1 g, 3.3 mmol) and incubated at room temperature for 24 hr in the dark. Compounds 1 and 2 were formed at the ratio of 1 : 1, followed by Sephadex LH 20 chromatography to purify each individual compound. After confirming the structure and purity, synthetic 1 and 2 were used for the following platelet aggregation and membrane fluidity experiments as well as isolates from the brownish scale of onion.

Platelet Aggregation —— The experiments were carried out according to the guidelines of the Japanese Pharmacological Society. Blood was collected from healthy adult donors (n = 4, aged 35–48 years), tested repeatedly in our laboratory, without any medication in the preceding three weeks after informed consent. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared from citrated plasma and the platelet count of PRP was adjusted to 300000/μl by diluting with PPP.[8,9] Platelet aggregation was analyzed by a 601 Hema Tracer aggregometer (Niko Bioscience, Tokyo, Japan) to monitor an increase of percent light transmission (% T) as a function of time, where PPP was 100% T and unstimulated PRP was 0% T, as reported previously.[9] In brief, after adjusting % T to 0, 20 μl of a sample solution was added to 170 μl of PRP so that the final concentrations of a crude MeOH extract and flavonoids (1, 2, 3, and 4) were 0.5–1.0 mg/ml and 0.5–5 mM, respectively. The tested samples were dissolved in 5%(v/v) aqueous dimethyl sulfoxide (DMSO) solution, which was added for control samples. The final concentration of DMSO in PRP was adjusted to be less than 0.5%(v/v) so as not to influence platelet aggregation. After 1 min, 10 μl of an aqueous solution of 50 μg/ml collagen (MC Medical, Tokyo, Japan), 60 μM adenosine 5′-diphosphate (ADP) (MC Medical), 5 U/ml thrombin (Sigma, St. Louis, MO, U.S.A.) or 40 μg/ml epinephrine (Daiichi-Seiyaku, Tokyo, Japan) was added to induce platelet aggregation. The time of inducer addition was defined as 0 min. Maximal % T (Tmax), area under curve (AUC) and slope of aggregation responses from 0 to 5 min were determined from the obtained aggregograms. Aggregation inhibition (%) was calculated by comparing Tmax, AUC and slope values with controls. In the disaggregation assay, 10 μl of an aqueous solution of 60 μM ADP was added to 170 μl of PRP to induce platelet aggregation. After the plateau of maximum aggregation had been reached, 20 μl of 5%(v/v) aqueous DMSO solution of 1 and 3 was added to PRP to give the final concentration of 2 mM for each and changes of % T were monitored for 5 min. The disaggregatory effects to dissociate the produced aggregates were evaluated by comparing % T at 10 min with % T at 5 min (maximum aggregation).

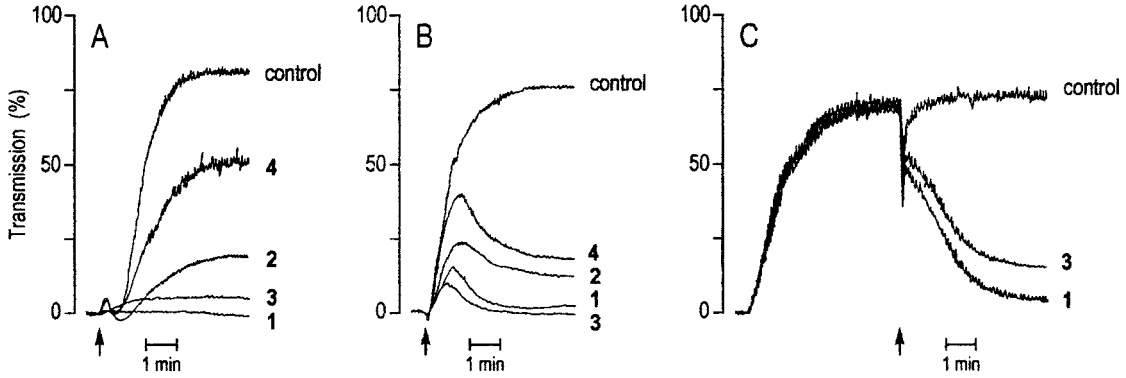

Fig. 2. Aggregograms Representing Anti-Aggregatory (A and B) and Disaggregatory (C) Effects of Flavonoidal Components on Human Platelets

A and B: After treatment of platelet-rich plasma with flavonoids 1, 2, 3 and 4 (2 mM for each) or solvent alone (control) for 1 min, collagen (A) or ADP (B) was added to induce platelet aggregation at the indicated time. C: After ADP-induced platelet aggregation responses became plateau, the produced aggregates were treated with flavonoids 1 and 3 (2 mM for each) or solvent alone (control) at the indicated time.

Membrane Effects —— Cholesterol, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1,2-dipalmitoylphosphatidylcholine (DPPC), and 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Wako (Osaka, Japan), Avanti Polar Lipids (Birmingham, AL, U.S.A.) and Molecular Probes (Eugene, OR, U.S.A.), respectively. The suspensions of liposomes with the lipid bilayer structure were prepared by sonicating the dry film of cholesterol and phospholipids (total lipids of 14.0 μmol) in 100 ml of 10 mM Tris–HCl buffer (pH 7.4, containing 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA and 7.5 mM sodium azide) as reported previously.[10,11] The lipid composition of liposomal membranes was 100 mol% DPPC, cholesterol : POPC = 20 : 80 (mol%) or cholesterol : POPC : DPPC = 40 : 40 : 20 (mol%). The sample solutions in DMSO were added to the liposome suspensions (total volume of 3.0 ml) so that the final concentrations of a crude MeOH extract and flavonoids (1, 2, 3, and 4) were 1.0 μg/ml and 0.25–10 μM, respectively. The concentration of DMSO in suspensions was adjusted to be less than 0.5%(v/v) so as not to influence the fluidity of liposomal membranes. After incubation at 37°C for 30 min, fluorescence polarization was measured by labeling liposomes with either DPH or TMA-DPH as reported previously.[12] Compared with control samples treated with DMSO alone, an increase of polarization values means the reduction of membrane fluidity (membrane rigidification).

Statistical Analysis —— All data are expressed as means ± SEM (n = 4–6). Statistical comparison was performed by Student's t-test and values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

A crude MeOH extract (0.5–1.0 mg/ml) showed the inhibitory effects on human platelet aggregation to produce 40.0–49.3% and 30.0–33.3% inhibition in collagen- and ADP-induced aggregation, respectively, compared with control Tmax and AUC. It also inhibited Tmax by 12.9–16.3% in thrombin- and epinephrine-induced aggregation. Based on these results, PRP was treated with 1, 2, 3 and 4 isolated from the anti-platelet extract. Their anti-aggregatory effects were comparatively studied using collagen and ADP as an inducer because the preparation of sample solutions needed more than 5%(v/v) DMSO which influenced the aggregation by thrombin and epinephrine. All of them effectively inhibited platelet aggregation at 2 mM (Fig. 2). When comparing inhibition % at the same molar concentration, 1 was the most effective on collagen-induced aggregation, followed by 3, 2 and 4 (Table 1). The concentrations to produce 50% inhibition (IC_{50}s) of Tmax, AUC and slope in collagen-induced aggregation were 1.02 ± 0.01, 1.05 ± 0.04 and 1.04 ± 0.02 mM for 1, 1.27 ± 0.10, 1.22 ± 0.08 and 2.24 ± 0.34 mM for 2, 1.09 ± 0.03, 1.08 ± 0.03 and 1.33 ± 0.20 mM for 3 and 3.99 ± 0.65, 4.86 ± 1.79 and 10.93 ± 1.47 mM for 4. The IC_{50}s of Tmax and AUC in ADP-induced aggregation were 1.66 ± 0.11 and 1.15 ± 0.05 mM for 1, 1.57 ± 0.11 and 1.38 ± 0.01 mM for 2, 1.30 ± 0.05 and 1.10 ± 0.02 mM for 3 and 3.08 ±

Table 1. Inhibitory Effects of Flavonoidal Components on Human Platelet Aggregation

Sample	Inhibition (%)					
	Collagen-induced aggregation			ADP-induced aggregation		
	Tmax	AUC	Slope	Tmax	AUC	Slope
1	98.2 ± 0.7	95.3 ± 3.2	95.9 ± 1.7	60.8 ± 3.7	87.1 ± 3.9	6.8 ± 0.4
2	79.8 ± 5.9	82.5 ± 5.3	47.2 ± 8.4	64.1 ± 4.2	72.3 ± 0.3	11.2 ± 0.4
3	92.4 ± 2.3	93.3 ± 2.8	60.8 ± 5.8	77.4 ± 3.1	90.5 ± 1.6	19.2 ± 1.3
4	28.1 ± 4.7	30.0 ± 6.8	9.8 ± 1.2	33.1 ± 3.2	67.2 ± 3.5	1.2 ± 0.1

After treatment of platelet-rich plasma with flavonoids 1, 2, 3 and 4 (2 mM for each) for 1 min, the aggregation was induced by collagen or ADP. Values are means ± SEM ($n = 4$–5).

0.30 and 1.50 ± 0.08 mM for 4. These IC_{50} values were slightly larger than those reported for quercetin and other flavonoids previously.[13,14] Such discrepancy in anti-platelet activity may be due to different aggregation systems that human platelets were used in the present study, but porcine[13] and rabbit platelets[14] in previous ones.

The ability not only to inhibit platelet aggregation but also to dissociate already aggregated platelets is important for a potent anti-platelet agent.[8,15,16] To evaluate the disaggregatory effects of flavonoidal components, more active 1 and 3 were introduced to PRP after the aggregation responses became plateau. They caused the disaggregation of ADP-produced aggregates at 2 mM (Fig. 2). The dissociated aggregates showed 77.6–93.2% decrease of % T after treatment with 1 and 3 for 5 min.

Membrane fluidity closely relates to the function of platelets,[17] and the modifiers of membrane fluidity are referred to as the potent inhibitors of primary and secondary platelet aggregation.[18] Membrane-active agents also have the property to dissociate platelet aggregates.[8] The membrane effects of onion scale components were evaluated using protein-free liposomes to focus on their interactions with membrane lipids as a mode of anti-platelet action. A crude MeOH extract (1.0 μg/ml), which showed the anti-platelet activity, increased both DPH and TMA-DPH polarization of 100 mol% DPPC liposomes most frequently used in membrane fluidity experiments. The ratio of DPH polarization increase to TMA-DPH polarization increase was 2.02 ± 0.04. DPH and TMA-DPH indicate a fluidity change in the hydrophobic regions and the hydrophilic regions of membranes, respectively.[12] The anti-platelet extract was considered to rigidify membranes by acting on the hydrocarbon core rather than the surface of lipid bilayers. Based on these results, the membrane effects of flavonoidal components were comparatively studied using DPH which was more suitable to determine the fluidity change and reflect the acting-site. DPH polarization measurement was performed at 0.25–10 μM because flavonoids of sub-mM and mM levels showed the possibility to cause artifactual polarization changes by their quenching effects on DPH fluorescence.[19] All of the anti-platelet components rigidified liposomal membranes with the potency being 1 > 2 > 3 > 4, while their rigidifying effects significantly depended on the membrane lipid composition (Table 2). Human platelet membranes show approximately 0.25 for the molar ratio of cholesterol to phospholipids as the major lipid components.[20] Anti-platelet flavonoids were more effective in rigidifying 20 mol% cholesterol and 80 mol% POPC liposomal membranes, indicating that they preferentially act on platelet membranes.

Anti-platelet agents and phytochemicals influence platelet aggregation by modifying membrane fluidity.[8,18,21] Various flavonoids interact with membrane lipids to change the fluidity.[22,23] The present study has revealed that the membrane-rigidifying effect is greatest in the order of 1, 2, 3 and 4 and that the anti-platelet activity of 3 is enhanced by dimerization as in 1 but reduced by 4′-glucosylation as in 4. Membrane rigidification appears to be one of pharmacological mechanisms underlying the anti-aggregatory and disaggregatory effects of the brownish scale of onion. Compared with aglycon (3), its 4′-glucoside (4) was less effective in inhibiting platelet aggregation and rigidifying liposomal membranes, which is accounted for by the decreased permeability into membrane lipids. In comparison of 1, 2 and 3, however, their anti-platelet and membrane activity were not necessarily correlated, suggesting the contribution of effects other than membrane rigidification. In addition to the interaction with membrane lipids, anti-platelet flavonoids including quercetin are known to inhibit cyclic nucleotide phosphodiesterase and cyclooxygenase,[24] an-

Table 2. Membrane-Rigidifying Effects of Flavonoidal Components on Liposomes Prepared with Cholesterol and Phospholipids of Different Molar Ratio

Sample	Concentration	DPH polarization change from control	
	(μM)	Cholesterol/POPC/DPPC (40 : 40 : 20)	Cholesterol/POPC (20 : 80)
1	10	0.0374 ± 0.0013**	0.1167 ± 0.0016**
	5	0.0256 ± 0.0014**	0.0655 ± 0.0009**
	2.5	0.0176 ± 0.0011**	0.0364 ± 0.0018**
	0.25	0.0010 ± 0.0009	0.0041 ± 0.0012**
2	10	0.0370 ± 0.0008**	0.1006 ± 0.0017**
	5	0.0223 ± 0.0009**	0.0431 ± 0.0013**
	2.5	0.0133 ± 0.0009**	0.0175 ± 0.0015**
	0.25	0.0003 ± 0.0012	0.0024 ± 0.0013*
3	10	0.0264 ± 0.0011**	0.0695 ± 0.0006**
	5	0.0119 ± 0.0013**	0.0229 ± 0.0013**
	2.5	0.0036 ± 0.0011**	–0.0011 ± 0.0010
	0.25	0.0003 ± 0.0012	0.0009 ± 0.0010
4	10	0.0065 ± 0.0009**	0.0152 ± 0.0015**
	5	0.0032 ± 0.0014*	0.0033 ± 0.0010*
	2.5	0.0000 ± 0.0011	0.0004 ± 0.0015
	0.25	0.0000 ± 0.0008	0.0006 ± 0.0014

Values are means ± SEM ($n = 5$). *$p < 0.05$ and **$p < 0.01$ compared with control.

tagonize the thromboxane receptor,[14] and reduce inositol phosphate production.[13]

The modification of membrane fluidity is also responsible for other different bio-activities including microbial growth-inhibition, cancer prevention, hepato-protection, anti-oxidation, *etc*. The brownish scale of onion containing membrane-active flavonoids is expected as a potent medicinal resource.

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